Annex IX

Reproducibility Analyses for LLNA: BrdU-ELISA with Decision Criterion of SI ≥ 1.5 or SI ≥ 2.0

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1.0 Test Method Reliability

Appendix C, Section 7 provides the reproducibility analyses for the LLNA: BrdU-ELISA using SI ≥ 1.6 to classify substances as sensitizers. This annex provides the reproducibility analyses using SI ≥ 1.5 or SI ≥ 2.0 to classify substances as sensitizers. The data used for the analyses in this annex are the LLNA: BrdU-ELISA results for the 31 substances (22 traditional LLNA sensitizers and nine traditional LLNA nonsensitizers) that were reviewed by the Panel at the public meeting on April 28-29, 2009. The decision criterion of SI ≥ 2.0 was used in the JSAAE interlaboratory validation study. The SI ≥ 2.0 criterion produced an accuracy of 87% (27/31), a false positive rate of 0% (0/9), and a false negative rate of 18% (4/22) when LLNA: BrdU-ELISA results were compared to the results of the traditional LLNA. The SI ≥ 1.5 criterion, which was one of the alternative SI criterion evaluated, produced an accuracy of 84% (26/31), a false positive rate of 33% (3/9), and a false negative rate of 9% (2/22) when LLNA: BrdU-ELISA results were compared to the results of the traditional LLNA.

1.1 Intralaboratory Reproducibility for SI ≥ 1.5

The test results for the LLNA: BrdU-ELISA were amenable to intralaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification, SI values, and EC1.5 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer in **Section 1.1.1** of this annex) and a CV analysis for the quantitative results (SI values and EC1.5 in **Sections 1.1.2** and **1.1.3** of this annex, respectively).

1.1.1 Intralaboratory Reproducibility – Qualitative Results

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included nine substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol was tested four times; diphenycyclopropenone and propylene glycol were tested three times; and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, and 4-phenylenendiamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table C-IX-1**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 8/9 substances were 100% concordant when SI ≥ 1.5 was used to classify substances as sensitizers; however, the concordant results for hexane were false positive with respect to the traditional LLNA. Discordant test results were noted for propylene glycol. The test results from Takeyoshi et al. (2005), which were tested at maximum concentrations of 10% and 50% were negative (SI = 1.20) and positive (SI = 1.57), respectively. The result from Takeyoshi et al. (2006) produced a negative result (SI = 0.91). All tests used AOO as the vehicle.

By comparison, the qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or 83% concordance) results in the traditional LLNA was reported as equivocal because SI increased with dose, but did not reach the criterion of SI ≥ 3.0. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was similar for LLNA: BrdU-ELISA (8/9) and the traditional LLNA (5/6).

Table C-IX-1 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times for SI ≥ 1.5

Substance	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.86	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopro-penone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.33	+	2004a
	30	3.83	+	2007a
	50	12.28	+	2005
	50	3.05	+	2006
	50	17.69	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	+	2005
	100	1.76	+	unpublished data
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.64	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Isoeugenol	10	8.36	+	2005
	10	2.36	+	2006, 2007b
	10	7.20	+	2005
	30	6.73	+	2007a
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	+	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (BrdU); SI = stimulation index.

[1] + = sensitizer; - = nonsensitizer.

1.1.2 Intralaboratory Reproducibility – SI ≥ 1.5

There were seven substances that were tested multiple times using the same concentrations by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were 10 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-IX-2**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

1.1.3 Intralaboratory Reproducibility – EC1.5

CV values were also calculated for the EC1.5 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003; 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculated EC1.5 values for the LLNA: BrdU-ELISA. The methods for calculating EC1.5 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC1.5 values for each test with SI values higher or lower than 2 and extrapolation was used to calculate EC1.5 values for tests with no SI values below 2. The equation for linear interpolation was:

$$EC1.5 = c + \left[\frac{(1.5 - d)}{(b - d)}\right] \times (a - c)$$

The linear interpolation equation uses the points immediately above and below SI = 2, with the (dose, SI) coordinates of (a, b) immediately above SI = 2 and (c, d) immediately below SI = 2. The equation for extrapolation was:

$$EC1.5_{ex} = 2^{\left\{\log_2(c) + \frac{(1.5-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

The extrapolation equation uses the two points immediately above SI = 2, with the coordinates of (a, b) for the point closest to SI = 2 and (c, d) for the higher point.

Table C-IX-2 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
2,4-Dinitrochlorobenzene	2	17.86	12.35	7.79	63	2005
		6.84				2006, 2007b
Diphenylcyclopropenone	10	9.34	10.48	1.61	15	2005; 2007b
		11.62				2007b
Eugenol	30	3.33	3.58	0.35	10	2004a
		3.83				2007a

continued

Table C-IX-2 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation (continued)

Substance	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	50	12.28	11.01	7.40	67	2005
		3.05				2006
		17.69				2007b
Hexane	50	1.89	1.64	0.36	22	2005
		1.38				Unpublished
Hexyl cinnamic aldehyde	12.5	1.88	1.74	0.21	12	2003
		1.59				2003
	25	2.44	2.42	0.02	1	2003
		2.41				2003
	50	3.64	3.78	1.25	33	2003
		5.90				2005
		3.64				2006
		2.72				2006
		3.02				2007b
Isoeugenol	10	8.36	5.09	3.15	80	2005
		7.20				2005
		2.36				2006, 2007b
		2.43				2007a
Propylene glycol	50	1.57	1.14	0.62	54	2005
		0.70				2006, 2007b

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation; SI = stimulation index.

As shown in **Table C-IX-3**, there were five EC1.5 values for hexyl cinnamic aldehyde, four EC1.5 values for eugenol, and two EC1.5 values for isoeugenol. The CV values were 37% for hexyl cinnamic aldehyde, 66% for eugenol, and 52% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility is based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2008a). Intralaboratory reproducibility is considered adequate when each test yields an ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value, 1.5, in this case) within 5% to 20% (ICCVAM 2008a). All five EC1.5 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-IX-3 Intralaboratory Reproducibility for the EC1.5 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	EC1.5	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	5.9	7.2	4.7	66	2004a
	11.0				2006
	10.7				2007a
	1.0				2007b
Hexyl cinnamic aldehyde	11.6	12.9	4.8	37	2003
	5.5				2003
	15.9				2006
	18.1				2006
	13.5				2007b
Isoeugenol	6.3	4.6	2.4	52	2006, 2007b
	2.9				2007a

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; EC1.5 = estimated concentration needed to produce a stimulation index of 1.5; SD = standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2, 4-dinitro-chlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table C-IX-4**).

Table C-IX-4 Intralaboratory Reproducibility for the EC3 of Tested Substances in the Traditional LLNA[1]

Substance	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	13 – 47
Isoeugenol	1	5	26
Hexyl cinnamic aldehyde	2	6	19-27
Eugenol	1	5	18

Abbreviations: CV = coefficient of variation; LLNA = murine local lymph node assay); EC3 = estimated concentration needed to produce a stimulation index of 3.

[1] From ICCVAM (1999).

For all three substances in common, the intralaboratory CV values for the EC1.5 values from LLNA: BrdU-ELISA tests were higher than those reported in ICCVAM (1999) for EC3 values from the traditional LLNA. The intralaboratory EC1.5 CV for the LLNA: BrdU-ELISA tests of eugenol was 66% vs. 18% for the CV of EC3 values reported by ICCVAM (1999). The intralaboratory EC1.5 CV for isoeugenol was 52% vs. 26% for the CV of EC3 values from ICCVAM (1999), and the intralaboratory EC1.5 CV for hexyl cinnamic aldehyde was 37% vs. 19% to 27% for the CV reported by ICCVAM (1999) for EC3 values.

1.2 Interlaboratory Reproducibility for SI ≥ 1.5

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multilaboratory validation study organized by the JSAAE (Kojima et al. 2008). The study design is described in **Appendix C, Section 7.2**. The LLNA: BrdU-ELISA test results from the study are amenable to interlaboratory reproducibility analyses for two endpoints: sensitizer or nonsensitizer classification and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer based on SI ≥ 1.5 in **Section 1.2.1** of this annex) and a CV analysis for the quantitative results (EC1.5 in **Section 1.2.2** of this annex).

1.2.1 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 1.5)

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with SI ≥ 1.5 at any dose were classified as sensitizers. The qualitative (i.e., sensitizer vs. nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during phase II of the JSAAE interlaboratory validation study is shown in **Table C-IX-5**. The qualitative comparison of LLNA: BrdU-ELISA results for nine substances tested in up to seven laboratories show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7). However, one of these substances, lactic acid, was misclassified as a nonsensitizer in all three laboratories. The concordance for isopropanol, the substance that produced discordant results among the laboratories, was 50% (3/6). The test of isopropanol at Laboratory 2 failed (SI = 1.09) because the concurrent positive control (SI = 1.29) failed the acceptance criterion of SI ≥ 2.The other six laboratories reported maximum SI values of 2.22, 0.98, 1.57, 0.94, 2.04, and 1.01. Thus, three tests were positive (SI ≥ 1.5) and three were negative (SI < 1.5). Isopropanol produces a nonsensitizer result in the traditional LLNA.

The Validation Management Team, which evaluated the reproducibility using SI ≥ 2 to identify sensitizers, considered the interlaboratory reproducibility to be acceptable (Kojima et al. 2008). Because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: BrdU-ELISA concordance.

Table C-IX-5 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochloro-benzene	+ (4.30)	+ (8.37)	+ (6.26)	+ (5.50)	+ (18.80)	+ (4.83)	+ (12.98)	7/7
Glutaraldehyde	+ (3.72)				+ (28.64)	+ (2.25)		3/3
Nickel sulfate			+ (2.58)	+ (4.53)			+ (2.66)	3/3
trans-Cinnamic aldehyde		+ (3.37)		+ (3.50)	+ (4.11)			3/3
Formaldehyde	+ (4.40)				+ (16.59)	+ (1.97)		3/3
Eugenol		+ (3.17)				+ (3.18)	+ (7.09)	3/3

continued

Table C-IX-5 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1] (continued)

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
Hexyl cinnamic aldehyde	+ (3.40)	-[3]	+ (2.87)	+ (3.34)	+ (13.50)	+[4] (3.27)	+ (3.84)	6/6
Isopropanol	+[2] (2.22)	-[3]	- (0.98)	+ (1.57)	- (0.94)	+[2,5] (2.04)	- (1.01)	3/6
Lactic acid			+ (1.80)	+ (1.89)			+ (2.53)	3/3
Methyl salicylate	- (1.43)	- (1.44)	- (1.40)					3/3

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] + indicates sensitizer result; - indicates nonsensitizer result using SI ≥ 1.5 to classify sensitizers. Maximum stimulation index values for each test are shown in parentheses.

[2] Test failed because concurrent positive control (SI = 1.29) failed the acceptance criterion (i.e., SI < 2). The positive control would have also failed if the acceptance criterion was SI ≥ 1.5. This isopropanol result was not included in the concordance analysis.

[3] Three mice tested at highest dose.

[4] Three mice per dose group.

1.2.2 Interlaboratory Reproducibility – EC1.5 Values

The SI values for each test were used to calculate EC1.5 values for each sensitizer according to the methods reported in **Section 1.1.3** of this annex. The EC1.5 values from each laboratory were used to calculate CV values for each substance. The resulting values are shown in **Table C-IX-6**. CV values ranged from 31% (*trans*-cinnamic aldehyde) to 95% (glutaraldehyde). The mean CV was 63%.

The ICCVAM LLNA *Performance Standards* indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2008a). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2008a). For 2,4-dinitrochloro-benzene, the EC1.5 values from four laboratories were outside the acceptable range. For hexyl cinnamic aldehyde, the EC1.5 values from two laboratories were outside the acceptable range. All values outside the acceptable ranges were below the low end of the range. This indicates that the discordance was due to the LLNA: BrdU-ELISA producing a more sensitive result.

Table C-IX-6 EC1.5 Values from the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA

Substance	Laboratory							Mean	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.058 (4.3 @ 1%)	***0.010 (8.37 @ 1%)***	***0.022 (5.99 @ 0.3%)***	***0.022 (5.50 @ 1%)***	***0.0022 (18.80 @ 0.3%)***	***0.015 (4.83 @ 0.3%)***	0.049 (12.18 @ 1%)	0.025	81
Hexyl cinnamic aldehyde	9.4 (3.4 @ 50%)	-[1] (1.83 @ 50%)	15.2 (2.87 @ 50%)	***4.1 (3.34 @ 50%)***	***3.5 (13.5 @ 50%)***	7.9[2] (3.27 @ 50%)	9.5 (3.84 @ 50%)	8.3	52
Glutaraldehyde	0.064	NT	NT	NT	0.031	0.21	NT	0.10	95
Nickel sulfate	NT	NT	1.5	0.5	NT	NT	0.6	0.8	65
trans-Cinnamic aldehyde	NT	1.7	NT	1.0	1.8	NT	NT	1.5	31
Formaldehyde	0.3	NT	NT	NT	0.2	0.6	NT	0.3	66
Eugenol	NT	12.5	NT	NT	NT	10.5	3.5	8.8	54

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2008a). Boldface italics show EC1.5 values that are outside of the acceptable range from the ICCVAM *LLNA Performance Standards*: 5% - 20% for hexyl cinnamic aldehyde and 0.025% - 0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are highest SI values achieved.

Abbreviations: CV =coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SI = stimulation index.

[1] Test failed because associated positive control failed acceptance criterion (i.e., SI < 2; vehicle control absorbance was unusually high). At SI = 1.29, the positive control would have failed even if the acceptance criterion was SI≥ 1.5. Result not included in the mean EC1.5 and CV.

[2] Three mice tested at highest dose.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC1.5 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7% to 84% for five substances tested in five laboratories (**Table C-IX-7**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for the EC1.5 from LLNA: BrdU-ELISA tests were greater than that for EC3 values from the traditional LLNA. The CV of 81% for EC1.5 values for 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27%, calculated from five EC3 values each, reported by ICCVAM (1999). The CV of 52% for EC1.5 values for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% CV for EC3 values reported by ICCVAM (1999). The CV of 54% for EC1.5 values for eugenol tested in the LLNA: BrdU-ELISA was greater than the CV of 42% for EC3 values reported by ICCVAM (1999).

Table C-IX-7 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance	Laboratory					CV (%)
	1	2	3	4	5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
SLS	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay; SLS = sodium lauryl sulfate.

[1] From ICCVAM (1999).

1.3 Intralaboratory Reproducibility for SI ≥ 2.0

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included nine substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol was tested four times; diphenylcyclopropenone and propylene glycol were tested three times; and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, and 4-phenylenendiamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table C-IX-8**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 9/9 substances were 100% concordant when SI ≥ 2.0 was used to classify substances as sensitizers.

By comparison, the qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or 83% concordance) results for the traditional LLNA was reported as equivocal because SI increased

with dose, but did not reach the criterion of SI ≥ 3.0. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was greater for the LLNA: BrdU-ELISA (9/9) than that for the traditional LLNA (5/6).

Table C-IX-8 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.86	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopro-penone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.33	+	2004a
	30	3.83	+	2007a
	50	12.28	+	2005
	50	3.05	+	2006
	50	17.69	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	-	2005
	100	1.76	-	unpublished data
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.64	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Isoeugenol	10	7.20	+	2005
	10	8.36	+	2005
	10	2.36	+	2006, 2007b
	30	6.73	+	2007a
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	-	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] + = sensitizer; - = nonsensitizer.

1.3.1 Intralaboratory Reproducibility – SI ≥ 2.0

There were seven substances that were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were 10 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-IX-2**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

1.3.2 Intralaboratory Reproducibility – EC2

CV values were also calculated for the EC2 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003; 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculate EC2 values for the LLNA: BrdU-ELISA. The methods for calculating EC2 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC2 values for each test with SI values higher or lower than 2 and extrapolation was used to calculate EC2 values for tests with no SI values below 2. The equation for linear interpolation was:

$$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

The linear interpolation equation uses the points immediately above and below SI = 2, with the (dose, SI) coordinates of (a, b) immediately above SI = 2 and (c, d) immediately below SI = 2. The equation for extrapolation was:

$$EC2_{ex} = 2^{\left\{\log_2(c) + \frac{(2-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

The extrapolation equation uses the two points immediately above SI = 2, with the coordinates of (a, b) for the point closest to SI = 2, and (c, d) for the higher point. As shown in **Table C-IX-9**, there were five EC2 values for hexyl cinnamic aldehyde, four EC2 values for eugenol, and two EC2 values for isoeugenol. The CV values were 73% for eugenol, 25% for hexyl cinnamic aldehyde, and 16% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility is based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2009). Intralaboratory reproducibility is considered adequate when each test yields an ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value; in this case, SI = 1.5) within 5% to 20% (ICCVAM 2009). Two of the five EC2 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-IX-9 Intralaboratory Reproducibility for the EC2 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	EC2	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	11.2	12.6	9.2	73	2004a
	23.6				2006
	1.2				2007b
	14.6				2007a
Hexyl cinnamic aldehyde	15.2	22.6	5.7	25	2003
	18.8				2003
	29.9				2006
	25.5				2006
	23.4				2007b
Isoeugenol	8.4	7.6	1.2	16	2006; 2007b
	6.7				2007a

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of 2; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2, 4-dinitrochlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table C-IX-4**).

For two of three substances, the intralaboratory CV values for the EC2 values from LLNA: BrdU-ELISA tests were higher than EC3 values for the same substances from the traditional LLNA reported in ICCVAM (1999). The intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of eugenol was higher that that reported by ICCVAM (1999) for the EC3 (73% vs. 18%). The intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of isoeugenol was greater than the EC3 CV from ICCVAM (1999) (26% vs. 16%). However, the intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of hexyl cinnamic aldehyde was within the EC3 CV range reported by ICCVAM (1999) (25% vs. 19% to 27%).

1.4 Interlaboratory Reproducibility for SI ≥ 2.0

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multi-laboratory validation study organized by the JSAAE (Kojima et al. 2008). The study design is described in **Appendix C, Section 7.2**. The LLNA: BrdU-ELISA test results from the study are amenable to interlaboratory reproducibility analyses for two endpoints: sensitizer or nonsensitizer classification and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer in **Section 1.4.1** of this annex) and a CV analysis for the quantitative results (EC2 values in **Section 1.4.2** of this annex).

1.4.1 Interlaboratory Reproducibility – Qualitative Results

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with SI ≥ 2.0 at any dose were

classified as sensitizers. The qualitative (sensitizer/nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during Phase II of the JSAAE interlaboratory validation study is shown in **Table C-IX-10**. The concordance results show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7) for seven substances. There were three discordant substances (formaldehyde, isopropanol, and lactic acid) for which interlaboratory concordance was 67% (2/3 or 4/6). One of the three laboratories reported an SI of 1.97 for formaldehyde, while the others produced SI > 2. Two of the six tests of isopropanol yielded SI ≥ 2.0 (SI = 2.0 and SI = 2.2); while the others yielded SI < 2. One of the three tests for lactic acid produced SI ≥ 2.0 (i.e., SI = 2.5), while the others yielded SI < 2.0. The Validation Management Team considered the interlaboratory reproducibility to be acceptable (Kojima et al. 2008). There were no traditional LLNA concordance data for comparison with the LLNA: BrdU-ELISA concordance because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999).

Table C-IX-10 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochloro-benzene	+ (4.30)	+ (8.37)	+ (6.26)	+ (5.50)	+ (18.80)	+ (4.83)	+ (12.98)	7/7
Glutaraldehyde	+ (3.72)				+ (28.64)	+ (2.25)		3/3
Nickel sulfate			+ (2.58)	+ (4.53)			+ (2.66)	3/3
trans-Cinnamic aldehyde		+ (3.37)		+ (3.50)	+ (4.11)			3/3
Formaldehyde	+ (4.40)				+ (16.59)	- (1.97)		2/3
Eugenol		+ (3.17)				+ (3.18)	+ (7.09)	3/3
Hexyl cinnamic aldehyde	+ (3.40)	-[3]	+ (2.87)	+ (3.34)	+ (13.50)	+3,[4] (3.27)	+ (3.84)	6/6
Isopropanol	+[2] (2.22)	-[3]	- (0.98)	- (1.57)	- (0.94)	+[2,3,5] (2.04)	- (1.01)	4/6
Lactic acid			- (1.80)	- (1.89)			+ (2.53)	2/3
Methyl salicylate	- (1.43)	- (1.44)	- (1.40)					3/3

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] + indicates sensitizer result; - indicates nonsensitizer result. Maximum stimulation index values for each test are shown in parentheses.

[2] Stimulation index (SI) ≥ 2 at lowest dose tested, but <2 at the higher doses. The Validation Management Team considered these to be nonsensitizer results (Kojima et al. 2008).

[3] Test failed because concurrent positive control failed (i.e., SI < 2). Result not included in the concordance analysis.

[4] Maximum SI = 1.97.

[5] Three mice tested at highest dose.

[6] Three mice per dose group.

1.4.2 Interlaboratory Reproducibility – EC2 Values

The SI values from the interlaboratory validation study were used to calculate EC2 values for each sensitizer according to the methods reported in **Section 1.3.3** of this annex. The EC2 values from each laboratory were then used to calculate CV values for each substance. The resulting values are shown in **Table C-IX-11**. CV values ranged from 20% (formaldehyde) to 101% (glutaraldehyde). The mean CV was 58%.

The ICCVAM LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). EC2 values from two laboratories were outside these ranges for both substances. Laboratory 2 and Laboratory 5 reported EC2 values that were lower than the specified acceptance range for 2,4-dinitrochlorobenzene (0.019% and 0.0025%, respectively). For hexyl cinnamic aldehyde, Laboratory 3 obtained an EC2 value of 24.0%, which was higher than the acceptance range, and Laboratory 5 obtained an EC2 value of 4.07%, which was lower than the acceptance range.

Table C-IX-11 EC2 Values from the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Mean	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.084 (4.3 @ 1%)	***0.019 (8.37 @ 1%)***	0.029 (5.99 @ 0.3%)	0.030 (5.50 @ 1%)	***0.0025 (18.80 @ 0.3%)***	0.025 (4.83 @ 0.3%)	0.053 (12.18 @ 1%)	0.035	76
Hexyl cinnamic aldehyde	16.2 (3.4 @ 50%)	-[1] (1.83 @ 50%)	***24.0 (2.87 @ 50%)***	9.36 (3.34 @ 50%)	***4.07 (13.5 @ 50%)***	13.0[2] (3.27 @ 50%)	14.2 (3.84 @ 50%)	13.5	50
Glutaraldehyde	0.18	NT	NT	NT	0.034	0.51	NT	0.24	101
Nickel sulfate	NT	NT	3.85	0.95	NT	NT	1.31	2.0	78
trans-Cinnamic aldehyde	NT	2.59	NT	1.63	2.79	NT	NT	2.3	27
Formaldehyde	0.41	NT	NT	NT	0.31	-[3]	NT	0.36	20
Eugenol	NT	19.1	NT	NT	NT	16.4	5.06	13.5	55

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Boldface italic EC2 values are outside of the acceptable range from the ICCVAM LLNA performance standards: 5% - 20% for hexyl cinnamic aldehyde and 0.025% - 0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are the highest SI values achieved.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of 2; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SI = stimulation index.

[1] Test failed because associated positive control failed (i.e., SI < 2; vehicle control absorbance was unusually high). Result not included in the mean EC2 and CV.

[2] Three mice tested at highest dose.

[3] Maximum SI = 1.97.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC2 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7% to 84% for five substances tested in five laboratories (**Table C-IX-12**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for LLNA: BrdU-ELISA were greater than those for the traditional LLNA. The CV of 76% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27%, calculated from five values each, reported by ICCVAM (1999). The CV of 50% for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% reported by ICCVAM (1999). The CV of 55% for eugenol tested in the LLNA: BrdU-ELISA was greater than the 42% reported by ICCVAM (1999).

Table C-IX-12 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance	Laboratory					CV (%)
	1	2	3	4	5	
2,4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

[1] From ICCVAM (1999).